# Short-Term Insulin Therapy and Normoglycemia

## Effects on erythrocyte lipid peroxidation in NIDDM patients

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**OBJECTIVE** — To evaluate erythrocyte lipid peroxidation (LPO) before and after an adaptive short-term insulin therapy in NIDDM patients who were chronically hyperglycemic.

**RESEARCH DESIGN AND METHODS** — Twenty-six patients with NIDDM (mean $HbA_{1c}$, 11.28%) aged 53.04 ± 2.03 years were submitted for 3 days to constant intravenous glucose and continuous insulin perfusion at an adaptable rate to maintain glycemia within the normal range. An evaluation of LPO at baseline and after euglycemic insulin therapy was determined by erythrocyte free and total malondialdehyde (MDA) levels, polyunsaturated fatty acid (PUFA) percentage, vitamin E and glutathione content, and the following antioxidant enzymatic activity determinations: glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT). Fasting serum glucose, $HbA_{1c}$, triglycerides, cholesterol, and HDL cholesterol levels were also determined at these time points.

**RESULTS** — At baseline, erythrocyte free and total MDA were significantly higher in NIDDM patients than in control subjects (11.14 ± 0.80 vs. 1.74 ± 0.11 nmol/g Hb [$P < 0.0001$] for free MDA; 18.04 ± 1.79 vs. 7.85 ± 0.55 nmol/g Hb [$P < 0.0001$] for total MDA). PUFAs, particularly C20:4 and C22:5, were increased (14.69 ± 0.34 vs. 12.03 ± 0.31 and 2.31 ± 0.04 vs. 1.71 ± 0.03 % of total fatty acids, respectively). Vitamin E and glutathione were reduced significantly (6.16 ± 0.61 vs. 14.84 ± 0.64 nmol/g Hb and 0.42 ± 0.04 vs. 0.97 ± 0.06 mmol/l, respectively). No difference was observed for the enzymatic activities. After euglycemic insulin therapy, triglycerides significantly decreased compared with baseline concentrations (1.55 ± 0.13 vs. 2.42 ± 0.22 mmol/l; $P < 0.001$), whereas other lipidic parameters were unchanged. Free MDA significantly decreased (8.60 ± 0.76 vs. 11.14 ± 0.80 nmol/g Hb [$P < 0.01$]), while vitamin E increased (7.93 ± 0.73 vs. 6.16 ± 0.61 nmol/g Hb [$P < 0.05$]). No difference was observed for PUFAs, glutathione, or total MDA.

**CONCLUSIONS** — The observed erythrocyte LPO in NIDDM decreased after a short-term adaptive insulin therapy. This decrease could be principally attributed to the normalized glycemia that reduces reactive oxygen species (ROS) production, which in turn may explain the increase in erythrocyte membrane vitamin E and the decrease in MDA. This study shows the value of a euglycemic environment in NIDDM to reduce LPO and, at long range, to minimize clinical diabetes complications.

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CAT, catalase; MDA, malondialdehyde; GPX, glutathione peroxidase; GSH, glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; LPO, lipid peroxidation; MDA, malondialdehyde; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid; TCA, trichloracetic acid.

Diabetes is associated with a markedly increased risk of atherosclerosis and cardiovascular disease (1). However, the mechanism by which diabetes leads to atherosclerosis and its complications has not been well established. Lipid peroxidation (LPO) has been implicated in the pathogenesis of naturally occurring or induced diabetes (2). Oxidant stress may be increased in diabetes owing to a hyperproduction of reactive oxygen species (ROS), such as $O_2^{\bullet}$, $OH^{\bullet}$, and $H_2O_2$, and/or a deficiency in the antioxidant defense system. The increased production of ROS has been attributed to protein glycation (3,4) and/or glucose autoxidation owing to a hyperglycemic environment (5). An impaired radical scavenger function has been linked to the decreased activity of enzymatic and nonenzymatic scavengers. Particularly, the action of superoxide dismutase (SOD), which causes the dismutation of $O_2^{\bullet}$ into $H_2O_2$, has been found to be decreased in the erythrocytes of diabetic rats (6). Similarly, a decrease in the action of SOD, glutathione peroxidase (GPX), and catalase (CAT), of which the latter two contribute to detoxify $H_2O_2$, have been observed in chronic diabetes (7). Among the nonenzymatic scavengers, vitamin E, the main intracellular antioxidant, has been found to be diminished in diabetes (8). Moreover, glutathione, which protects hemoglobin and some thiol-dependent enzymes or membrane proteins from oxidative damage, has been found to be reduced (9,10).

Several recent studies have confirmed the beneficial effects of short-term rigorous insulin therapy on glucose homeostasis in poorly controlled NIDDM patients (11–13), but less is known about the effects on erythrocyte oxidative stress.

Some authors have demonstrated that insulin treatment can increase enzymatic activities (6,14). Groop et al. (15) have demonstrated with a euglycemic insulin clamp that the regulation of intracellular fatty acid oxidation is complex and dependent in part on insulin concentration.

The present study aims to determine the basal level of erythrocyte LPO in chronically hyperglycemic NIDDM and to evalu-

Table 1—*Clinical and metabolic characteristics of the study subjects*

| | Control subjects | NIDDM patients |
|---|---|---|
| *n* | 24 | 26 |
| Sex (M/F) | 15/9 | 19/7 |
| Age (years) | 46.12 ± 2.04 | 53.04 ± 2.03 |
| Fasting plasma glucose (mmol/l) | 4.87 ± 0.03 | 13.88 ± 0.76 |
| $HbA_{1c}$ (%) | 5.30 ± 0.11 | 11.28 ± 0.49 |
| BMI (kg/m$^2$) | 24.20 ± 0.90 | 28.50 ± 1.30 |

Data are *n* or means ± SE.

ate the effect of a short-term insulin therapy on erythrocyte LPO. The extent of the peroxidation reaction was evaluated by measuring several LPO parameters (e.g., polyunsaturated fatty acids [PUFAs], the direct targets of free radicals, and malondialdehyde [MDA], which is formed from the breakdown of PUFAs) and by evaluating the antioxidant enzymatic and nonenzymatic systems.

## RESEARCH DESIGN AND METHODS

### Patients and sample collection

Twenty-six adult NIDDM patients (19 men and 7 women, aged 53.04 ± 2.03 years [mean ± SE]) were selected for the study from the Department of Internal Medicine at Bordeaux Hospital.

Diabetes was diagnosed in these patients according to the World Health Organization criteria (16). The clinical and metabolic data of the group are shown in Table 1. Fasting plasma glucose levels were 13.88 ± 0.76 mmol/l on the morning after the admission for chronic hyperglycemia. Nonenzymatically glycosylated hemoglobin levels were 11.28 ± 0.49%. Diabetes was known for 5.67 ± 0.26 years, and all patients were treated with an oral antidiabetic agent.

The control group was composed of 24 healthy adults (15 men and 9 women, aged 46.12 ± 2.04 years). No patients or control subjects were taking any drugs that could influence the parameters under investigation, particularly vitamins A, E, or C. All subjects gave consent for the study.

Blood samples were drawn between 8:00 and 9:00 A.M. after an overnight fast from the cubital vein of control subjects and NIDDM patients at baseline and after euglycemic insulin therapy. Blood was stored in Vacutainer tubes (Becton-Dickinson, Meylan, France) without anticoagulant for glucose and lipidic parameters, with EDTA for $HbA_{1c}$, antioxidant enzymes, and glutathione determination, and with heparin for MDA and PUFA determination. For antioxidant enzymes, the Vacutainers were immediately put on ice, and the hemolysates were analyzed in the following hours.

### Experimental insulin protocol

After blood sample collection, a 150-g/24-h glucose infusion was started and continued for three days and was associated with intravenous insulin infusion at a variable rate adapted every 2 h according to the capillary blood glucose level. This level was controlled in the laboratory at every three controls. The insulin infusion rate was 0.056 ± 0.01 U · kg$^{-1}$ · h$^{-1}$ at the beginning of the protocol and was decreased in relation to the blood glucose level to obtain a normoglycemia value. At the end of the three days, the mean insulin rate was 0.031 ± 0.02 U · kg$^{-1}$ · h$^{-1}$. During the three-day protocol, meals were served without carbohydrates, which were infused. Normoglycemia was obtained within the first 6 h and then remained constant for the following 75 h. Glycemic levels never exceeded 6.8 mmol/l and never dropped below 3.2 mmol/l.

### LPO parameters

**Fatty acid determination.** As previously reported, fatty acid composition was determined by gas chromatograph after transformation into isopropylic esters, using Carlo Erba 6000 G (Fisons Instruments, Paris, France) gas chromatography (17).

Peak identification was made by comparison with reference fatty acid esters (Sigma, St. Louis, MO), and peak areas were measured with a DP 700 automatic integrator (Fisons Instruments). Results for each fatty acid are expressed as a percentage of the total fatty acids.

**MDA assays.** MDA assays were performed on erythrocytes by high-performance liquid chromatography (HPLC) according to a modified technique of Carbonneau et al. (18). Briefly, 2 ml of packed erythrocytes were suspended in 2 ml of 0.9% NaCl, and a sample was taken for hemoglobin analysis. The erythrocyte suspension was then frozen at −20°C during 24 h. After thawing at 37°C for 10 min, free and total MDA were immediately performed as follows. For total MDA, 1 ml of hemolyzed erythrocytes was incubated with NaOH at 60°C for 30 min. The hydrolyzed sample was then acidified with 10% trichloroacetic acid (TCA) to precipitate proteins. After centrifugation at 1,000*g* for 10 min, 300 µl of supernatant were treated with 50 µl of 0.5% thiobarbituric acid (TBA), and the color reaction was activated by heating for 30 min at 100°C. For free MDA, 500 µl of hemolyzed erythrocytes was treated with 50 µl of TCA 10%, and after centrifugation at 1,000*g* for 10 min, 300 µl of supernatant was treated with TBA as previously described for total MDA. The MDA-TBA complex was isolated by HPLC using a C18 µBondapack column (Waters, Ontario, Canada) with a 60/40 (vol/vol) mixture of 10 mmol/l phosphate buffer, pH 5.8, and methanol as a mobile phase. The flow rate of the solvent was 1.5 ml/min. The fluorometric detector was set at excitation 515 nm and emission at 532 nm. The standard used was 1,1,3,3-tetraethoxypropane (Aldrich-Chemic, Steiheim, Germany).

**Vitamin analysis.** As previously described, erythrocyte α-tocopherol analysis was performed by an HPLC procedure using a C18 µBondapack column (Waters) with methanol/water (98.5/1.5 [vol/vol]) as a mobile phase at a flow rate of 2 ml/min (19). The spectrophotometer was set at 290 nm.

**Enzymatic activity determination.** Erythrocyte SOD and GPX activities were determined using a Randox test combination (Randox, Crumlin, U.K.). SOD and GPX enzymes were measured on a Kontron spectrometer at 505 and 340 nm, respectively, as previously reported (20,21). The results are expressed in SOD or GPX units per gram of hemoglobin.

CAT activity was assessed according to Aebi (22). This method is based on the decomposition of $H_2O_2$ by CAT. The decrease in the absorbance at 240 nm was measured at 20°C. CAT activity was expressed in units per milligram of hemoglobin.

**Determination of glutathione levels.** Glutathione levels (the reduced form GSH and the oxidized form GSSG) were determined according to Coutelle (23). The assay was performed on a hemolyzated and deproteinized sample of whole blood.

**Table 2—*Evolution of biochemical parameters at baseline and after the insulin therapy***

| | Baseline | Final |
|---|---|---|
| Glucose (mmol/l) | 13.88 ± 0.76 | 5.43 ± 0.29* |
| Total cholesterol (mmol/l) | 5.44 ± 0.21 | 5.12 ± 0.20 |
| HDL cholesterol (mmol/l) | 0.90 ± 0.03 | 0.90 ± 0.03 |
| LDL cholesterol (mmol/l) | 3.41 ± 0.16 | 3.52 ± 0.17 |
| Triglycerides (mmol/l) | 2.42 ± 0.22 | 1.55 ± 0.13† |

Data are means ± SE. *Significant difference compared with baseline values at $P < 0.0001$. †Significant difference compared with baseline values at $P < 0.001$.

Specific measurement of GSSG was done after the inhibition of the GSH form under the action of 2 vinyl pyridine, and the GSH value was obtained by the difference between total glutathione and the GSSG form. Results are expressed in millimoles per liter.

**Biochemical parameters.** Capillary blood glucose level was performed in patients with a test strip (Lifescan, Milpitas, CA).

Plasma glucose, cholesterol, and HDL-cholesterol were measured in patients and control subjects on a multiparameter automate (Baxter, Paris, France). LDL-cholesterol was obtained using Friedwald calculation. $HbA_{1c}$ (percent) was determined at 415 nm by affinity chromatography on microcolumns (Biorad, Paris, France).

### Statistical analysis

Statistical analysis was performed on a Macintosh computer using Statview software package (Cricket Software, London, U.K.). Differences between NIDDM patients at baseline versus control subjects were compared by the two-tailed Mann-Whitney test. Differences between NIDDM patients after insulin therapy and at baseline were compared using Wilcoxon's test. $P < 0.05$ was considered statistically significant.

## RESULTS

### Biochemical data

Table 2 shows the evolution of biochemical parameters in NIDDM patients at baseline, compared with control subjects and after insulin therapy. At baseline, we noted the known dyslipidemia usually observed in NIDDM patients, particularly hypertriglyceridemia ($P < 0.001$ vs. control subjects), owing to the accumulation of VLDLs and their remnants and low levels of HDLs ($P < 0.001$ vs. control subjects).

The efficiency of the experimental insulin protocol was indicated by the significant decrease of glycemic levels ($P < 0.0001$ vs. baseline), which reached the control levels.

Similarly, insulin therapy rapidly normalized triglyceride levels, whereas other lipid parameters remained unchanged.

### Free and total MDA

At baseline, erythrocyte free and total MDA was significantly higher in NIDDM patients versus control subjects ($P < 0.0001$), therefore indicating increased erythrocyte LPO (Table 3). After the 3-day euglycemic insulin therapy, a significant decrease in free MDA was observed, compared with baseline ($P < 0.01$), even though this level remained higher than in control subjects ($P < 0.001$). Total MDA had a tendency to decrease after euglycemic insulin therapy, but the difference was not significant.

### Vitamin E levels

Vitamin E was significantly lower in erythrocytes of NIDDM patients than in control subjects ($P < 0.0001$) (Table 3). After the 3-day euglycemic insulin therapy, this level significantly increased ($P < 0.05$), compared with baseline, without reaching the values of control subjects.

### Determination of enzymatic activity and glutathione levels

As shown in Table 4, no significant difference was observed in the three enzyme activities either at baseline versus control subjects or after the insulin therapy when compared with baseline. On the other hand, GSH values were dramatically lower in NIDDM patients at baseline, compared with control subjects ($P < 0.0001$), whereas GSSG values were unchanged. After the 3-day euglycemic insulin therapy, GSH and GSSG values remained unchanged (Table 4).

### Erythrocyte lipid composition

The composition of fatty acids in erythrocytes of the NIDDM patients is shown in Fig. 1. Before insulin therapy, saturated fatty acids C16:0 were lower ($P < 0.05$), whereas PUFAs were higher, particularly C20:4 ($P < 0.001$) and C22:5 ($P < 0.01$), than in control subjects. After insulin therapy, no significant difference was observed in the percentage of fatty acids between the final and baseline values.

**CONCLUSIONS** — Before insulin therapy, erythrocyte MDA (free and total) levels were found to be higher in NIDDM patients than in control subjects. Since MDA is the currently used marker of peroxidative damage (24), our results, which are in accordance with other studies, provide evidence for increased LPO in the erythrocytes of NIDDM patients (25–27). However, we found that diabetic erythrocyte membranes contained a significantly higher proportion of arachidonic acid C20:4 and docosapentaenoic acid C22:5, compared with normal erythrocyte membranes. These results, which are also in agreement with other studies (28), suggest that the high level of PUFAs indicated an abnormal lipid content of erythrocyte membranes in diabetic patients. Because of their multiple double bond configuration, PUFAs are more susceptible than saturated or monounsaturated fatty acids to free radical peroxidation (29). Thus, diabetic erythrocyte membranes are more readily

**Table 3—*Erythrocyte malondialdehyde and vitamin E values in control subjects and in NIDDM patients at baseline and after insulin therapy***

| | Vitamin E (nmol/g Hb) | Free MDA (nmol/g Hb) | Total MDA (nmol/g Hb) |
|---|---|---|---|
| Control subjects ($n$ = 24) | 14.84 ± 0.64 | 1.74 ± 0.11 | 7.85 ± 0.55 |
| NIDDM baseline ($n$ = 26) | 6.16 ± 0.61* | 11.14 ± 0.80* | 18.04 ± 1.79* |
| NIDDM final ($n$ = 26) | 7.93 ± 0.73† | 8.60 ± 0.76‡ | 13.12 ± 0.81 |

Data are means ± SE. *Significant difference compared with controls values at $P < 0.0001$. †Significant difference compared with baseline values at $P < 0.05$. ‡Significant difference compared with baseline values at $P < 0.01$.

Table 4—*Antioxidant enzymatic activities and glutathione values in control subjects and in NIDDM patients at baseline and after insulin therapy*

| | *n* | SOD (U/g Hb) | GPX (U/g Hb) | CAT (U/mg Hb) | GSH (mmol/l) | GSSG (mmol/l) |
|---|---|---|---|---|---|---|
| Control subjects | 24 | 792 ± 33 | 38.80 ± 2.15 | 43.00 ± 2.50 | 0.97 ± 0.06 | 0.30 ± 0.02 |
| NIDDM baseline | 26 | 737 ± 37 | 43.50 ± 3.00 | 50.50 ± 5.10 | 0.42 ± 0.04* | 0.28 ± 0.02 |
| NIDDM final | 26 | 733 ± 37 | 38.50 ± 3.00 | 49.00 ± 3.40 | 0.41 ± 0.03 | 0.30 ± 0.03 |

Data are *n* or means ± SE. *Significant difference compared with control values at $P < 0.0001$.

oxidizable than those of control subjects, owing to their greater unsaturated fatty acid contents, especially arachidonic acid and docosapentaenoic acid.

Oxidative stress could result from an overproduction of ROS and/or from a decreased scavenger system efficiency. In diabetic patients, an excessive ROS production rate has been found (30) to be related to poor metabolic control and hyperglycemia (31,32). With regard to the antioxidant system status, we found that vitamin E, the main intracellular antioxidant, was significantly lower in the erythrocytes of NIDDM patients, compared with control subjects. This lower level could be attributed either to the decrease in serum level, owing to excessive production of free radicals in NIDDM patients, or to its consumption as chain-breaking antioxidant, owing to the presence of great oxidizable substrate in erythrocytes. Vitamin E efficiency has been demonstrated in vitro in suppressing oxidative damage in reconstituted liposomes prepared from erythrocyte membrane lipids of diabetic subjects (28). On the other hand, SOD activity, which directly scavenged $O_2^{\cdot}$ outside the membrane, was not different in erythrocytes of diabetic patients or control subjects. Consequently, as it is known that $O_2^{\cdot}$ generated outside the membrane can freely diffuse across the membrane (33), it seems that $O_2^{\cdot}$ induces oxidative stress within the membranes where lipids are more unsaturated than the lipids of normal erythrocytes, thus explaining the decrease in vitamin E.

No change in the activity of GPX was observed in diabetic patients, whereas a great decrease in GSH was found, in agreement with other studies (34–36). Glutathione is present in almost all cells and plays an important role in cellular protection against oxidation by free radicals and reactive oxygen intermediates (37,38). The glutathione redox cycle is regulated by the intracellular contents of GSH and GSSG and by GPX and glutathione reductase. A decrease in the activity of glutathione reductase leading to the decrease in the regenerating activity of GSSG to GSH was reported in streptozocin-induced diabetic rats (40). Moreover, in diabetic subjects, the increased sorbitol synthesis causes NADPH depletion, which when deficient also limits the reduction of GSSG to GSH (39). Therefore, the great decrease in GSH may profoundly impair free radical scavenging activity, resulting in exacerbated cell damage after exposure to free radicals generated by glucose autoxidation.

After a short-term insulin therapy that normalized glycemia, free MDA significantly decreased ($P < 0.01$) and vitamin E increased ($P < 0.05$), compared with baseline, whereas the PUFA percentage and GSH levels did not change. No correlation between insulin-mediated inhibition in oxidative stress and baseline glycosylated hemoglobin was found, suggesting that baseline metabolic control does not occur in the decrease of oxidative stress. It is known that the fatty acid component of cell membranes is a cellular factor that may influence the action of insulin. Increasing the content of PUFAs within cell membranes in culture increases membrane fluidity, the number of insulin receptors, and thus the action of insulin (41,42). Moreover, insulin sensitivity has been correlated positively with the percentage of C20:4 and the unsaturation index (43). Thus, the high levels of erythrocyte PUFAs observed in our NIDDM patients may improve the action of insulin. Moreover, several studies have shown that insulin can act on LPO by several mechanisms. First, a direct relationship has been demonstrated between plasma $O_2^{\cdot}$ levels and insulin action in NIDDM patients (44). Secondly, insulin can suppress intracellular lipid oxidation by a direct inhibitory effect (15).

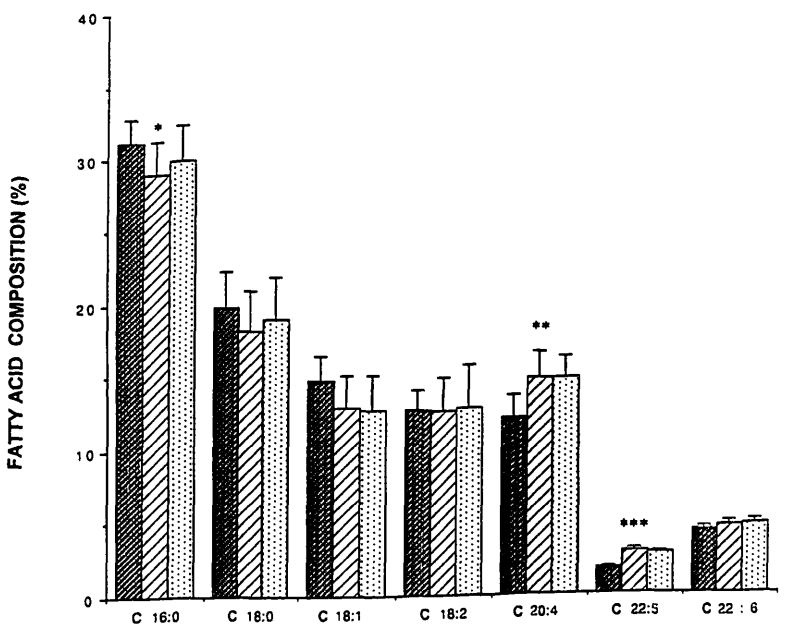


**Figure 1**—*Bar graph showing the erythrocyte fatty acid percentage in control subjects (■) and in NIDDM patients at baseline (▨) and after insulin therapy (▩). Data are means ± SD. Significant differences compared with values for the diabetic group at baseline and for the control group at* *$P < 0.05$, **$P < 0.001$, *and* ***$P < 0.01$.

However, in our protocol therapy, the insulin dose was immediately adjusted with a concomitant infusion of glucose to maintain glycemia at a normal level. Other authors have suggested a direct relationship between the level of glycemia and erythrocyte LPO. Increases in erythrocyte membranes LPO and MDA lipid adduct formation were observed when they were incubated only with glucose solution (≥5 mmol/l), and this effect was confirmed solely to glucose, not to plasma or other sugars (45). Moreover, it has been demonstrated that hyperglycemia, via the activation of the sorbitol pathway and the formation of triose phosphate autoxidizable, led to the generation of two reactive species α-oxaldehyde and $H_2O_2$ (46). Glycemia rapidly normalized after the beginning of the protocol may decrease the production of ROS by reducing glucose autoxidation (5,47), which could explain in part the increase in vitamin E content. However, GSH levels were not restored by insulin protocol. In diabetic rabbits, the concentration of glutathione and its related enzyme activities in the liver were reduced at baseline and subsequently restored by treatment with insulin (48). In our study, the short duration of the therapy did not lead to an increase in the GSH level via the polyol pathway. It could be that a longer treatment would be necessary to increase the GSH levels, as has been demonstrated in chronic insulin treatment (49).

Thus, our results suggest that *1*) elevated levels of PUFAs in erythrocyte membranes in NIDDM patients increase the LPO, owing to an excessive production of ROS and a decrease in GSH, and 2) short-term adjustment with insulin therapy to maintain glucose homeostasis decreases LPO. This decrease could be attributed principally to the normalized glycemia level in reducing ROS production and/or to the improvement in insulin action, since a lower insulin infusion rate is related to the same plasma glucose concentration target.

The present study confirms the increased erythrocyte membrane LPO in NIDDM patients. Moreover, the rapid normalization of glycemia with the 3-day insulin protocol could be of interest in decreasing LPO within diabetic erythrocyte membranes.

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